Paracrine signaling through the epithelial estrogen receptor α is required for proliferation and morphogenesis in the mammary gland

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Estradiol is a major regulator of postnatal mammary gland development and thought to exert its effects through estrogen receptor α (ERα) expressed in the mammary gland stroma and epithelium. Previous studies, however, were confounded by the use of an ERα mutant strain that retains some of the protein with transactivation activity. Here, we use an ER$\alpha^{-/-}$ mouse strain in which no ERα transcript can be detected to analyze mammary gland development in the complete absence of ERα signaling. The ER$\alpha^{-/-}$ females show no development beyond a rudimentary ductal system. By grafting ER$\alpha^{-/-}$ epithelium or stroma in combination with ERα WT stroma or epithelium, we show that the primary target for estradiol is the mammary epithelium, whereas a direct response of the mammary stroma is not required for mammary gland development to proceed normally. Mammary glands reconstituted with ER$\alpha^{-/-}$ mammary epithelium exposed to pregnancy hormones show increased transcription of milk protein genes, indicating that ERα signaling is not an absolute requirement for a transcriptional response to pregnancy hormones. When ER$\alpha^{-/-}$ mammary epithelial cells are in close vicinity to ERα WT cells, they proliferate and contribute to all aspects of mammary gland development, indicating that estradiol, like progesterone, orchestrates proliferation and morphogenesis by a paracrine mechanism, affecting nearby cells in the mammary epithelium.

transactivation function 1 | epithelial–mesenchymal interactions | branching | hormonal control | tissue recombination

The mammary gland is the only organ that undergoes most of its development postnatally. During embryogenesis, a rudimentary ductal system develops that grows isometrically with the rest of the body during the first weeks of life. At the onset of puberty, the ducts extend from the nipple area into a pad of fatty connective tissue that lies under the skin. The tips of the ducts enlarge to form club-shaped structures called terminal end buds (TEBs), which contain highly proliferative cells (1). Once the ducts have penetrated the fat pad through dichotomous branching, the complexity of the milk duct system increases with repeated estrous cycles through the growth of side branches. Ductal side branching becomes more extensive during pregnancy. Subsequently, alveoli bud off the ducts and differentiate to become sites of milk production (2).

Endocrine ablation and replacement studies in rodents established the female reproductive hormones estrogen, progesterone, and prolactin as master regulators of this postnatal development (3, 4). Tissue recombination experiments using mice in which both alleles of the progesterone receptor (5) or the prolactin receptor (6) have been deleted have shown that the epithelial progesterone receptor is required for ductal side branching (7), whereas prolactin signaling is required in the epithelium for alveologenesis and differentiation into milk-producing cells (6). Estrogen was shown to trigger ductal elongation during puberty (1); this induction was thought to be mediated by both stromal and epithelial estrogen receptor α (ERα) (8, 9).

Estrogens are ovarian hormones that regulate diverse physiological responses, including normal functioning of the reproductive and cardiovascular systems and bone metabolism (10–12). Estrogens act primarily through two nuclear estrogen receptors, ERα and ERβ, which are ligand-inducible transcription factors that modulate gene transcription by binding as hormone receptor complexes to specific DNA sequences (hormone response elements) in target promoters (13, 14). In addition, nongenomic actions of estrogens have been described (15).

Both ERα and ERβ are expressed in the mammary gland (16–18). Mouse models have been generated in which either the ERα or the ERβ locus was inactivated in the germ line. Deletion of ERβ (19) interfered with terminal differentiation of the mammary gland but did not affect ductal growth (20).

Consistent with estrogen triggering ductal outgrowth, deletion of ERα results in a rudimentary ductal system that fails to grow out (21). The interpretation of this mammary gland phenotype was complicated by the fact that estradiol influences the mammary gland at different levels. Estradiol stimulates the secretion of prolactin by the anterior pituitary gland (22) and suppresses the release of gonadotropins (23); consequently, the impairment of mammary gland development in the mutant mice may be due to the mammary gland's inability to respond adequately to estradiol itself, or it may be secondary to endocrine abnormalities. Different tissue recombination studies were conducted to resolve these issues. A study in which embryonic mammary gland components were recombined and transplanted under the kidney capsule concluded that mammary gland development required ERα expression in the stroma (24). Another series of experiments, based on grafts of mammary gland tissue from adult mice that were hormonally stimulated, indicated that both epithelial and stromal ERαs were necessary during mammary gland development (9).

However, all of these experiments are confounded by the presence of a truncated ERα protein in the ER$\alpha^{-/-}$ strain used (25). The ER$\alpha^{-/-}$ gene was disrupted by an insertion of the neomycin-resistance gene into the first coding exon. Alternative splicing gives rise to a transcript that generates an ERα protein lacking the N-terminal ligand-independent transactivation function-1 but retaining the ligand-dependent transactivation function-2 localized in the C-terminal ligand-binding domain (26, 27). This protein still possesses substantial transactivation capability (26, 27).

Here, we use a more recently developed ER$\alpha^{-/-}$ mouse strain that was generated by targeting exon 3, in which no ERα transcript can be detected (28), to analyze mammary gland development in the complete absence of ERα signaling. We find that ERα is required in the mammary epithelium for ductal outgrowth and

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Abbreviations: ERα, estrogen receptor α; MEC, mammary epithelial cell; TEB, terminal end bud.

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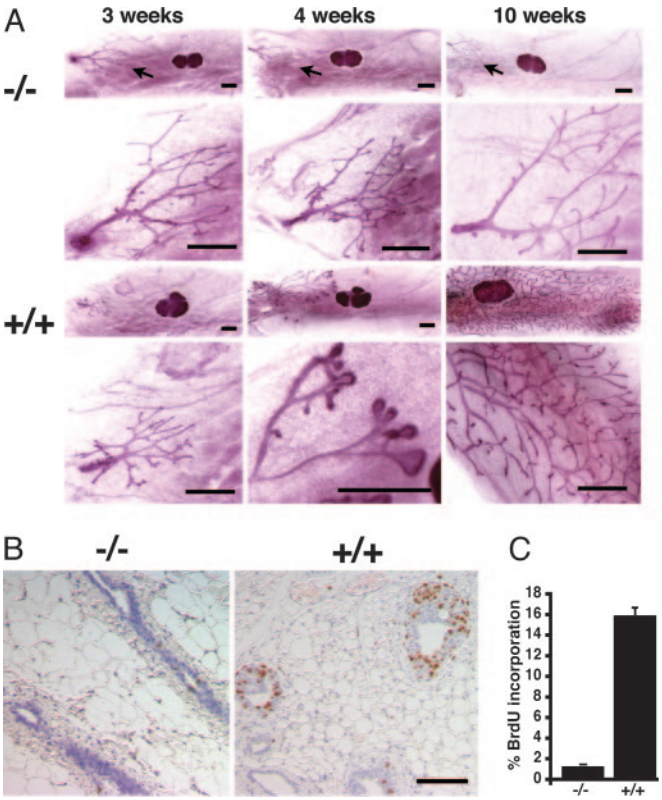

Fig. 1. Mammary gland development in ER$\alpha^{-/-}$ mice. (*A*) Whole-mounted mammary glands of mutant and WT females at different developmental stages. First and third rows show inguinal glands with lymph node. Second and fourth rows show higher magnification of the ductal tree. (Scale bars: 1 mm.) (*B*) Histological sections of mammary glands stained with an anti-BrdUrd antibody. (*C*) The percentage of BrdUrd-positive MECs is plotted in the bar graph. A total of 2,000–3,000 cells were counted in three different sections from different mice.

subsequent morphogenetic events but not for the transcriptional response to pregnancy hormones and that estradiol acts by a paracrine mechanism to orchestrate the proliferative and morphogenetic events in the mammary epithelium.

Results

Mammary Gland Development in ER$\alpha^{-/-}$ Mice. To assess the effects of systemic deletion of the two ERα alleles on mammary gland development, we analyzed the mammary glands of a series of ER$\alpha^{-/-}$ female mice along with those of WT littermates at different developmental stages. Up to puberty (3 weeks), there was no difference between mutant and WT littermates (Fig. 1*A*). Histological analysis revealed that the tissue architecture of the rudimentary ductal tree is normal (data not shown). At 4 weeks of age, when puberty had occurred, TEBs formed in the glands of WT mice, and ductal elongation ensued. We failed to detect TEBs in ER$\alpha^{-/-}$ female mice (Fig. 1*A*). Analysis of older virgin mice showed that even when the ductal tree in WT mice had fully penetrated the mammary fat pad, in the absence of ERα, no ductal elongation occurred (Fig. 1*A*). Thus, consistent with previous reports, prepuberal mammary gland development is not overtly impaired in the ER$\alpha^{-/-}$ mice, but the prepuberal rudimentary ductal system fails to develop farther (21). To assess whether this failure was a result of decreased proliferation or increased apoptosis, we determined the proliferative and apoptotic indices of the mammary glands by BrdUrd incorporation and immunohistochemistry for cleaved caspase 3 (Fig. 1*B* and data not shown). WT mammary glands at 4 weeks of age showed on average 15.8% of cells in S phase, whereas in ER$\alpha^{-/-}$ mammary glands only 1.2% of counted cells incorporated BrdUrd (Fig. 1*C*). Immunostainings for activated caspase 3 revealed <1% of the cells positive in mammary glands of both genotypes (data not shown), suggesting that the developmental defect is not a consequence of increased apoptosis but results from impaired proliferation.

The Role of ERα in the Mammary Epithelium. To assess whether the developmental defect was intrinsic to either the ER$\alpha^{-/-}$ mammary epithelium or the mammary stroma or whether it was secondary to endocrine disturbances of the mutant females, we performed tissue recombination experiments using mammary gland tissue from ER$\alpha^{-/-}$ females (28) and their WT littermates. In 3-week-old mice, the inguinal glands can be cleared of endogenous epithelium by surgically removing the nipple near half, because the ductal tree fills only a minor portion of the fat pad. Mammary epithelial cells (MECs) that are introduced into the remaining "cleared" fat pad will give rise to a new ductal system. MECs can grow out from a piece of breast tissue that is implanted (29) or from single-cell suspensions injected into the fat pad (30).

One cleared inguinal fat pad was engrafted with MECs derived from ER$\alpha^{-/-}$ female mice, the contralateral one with MECs derived from a WT littermate. Three-week-old, prepuberal donors were used to exclude differences between the two genotypes secondary to different hormone exposures. To ensure that comparable amounts of mammary epithelium were engrafted, we used

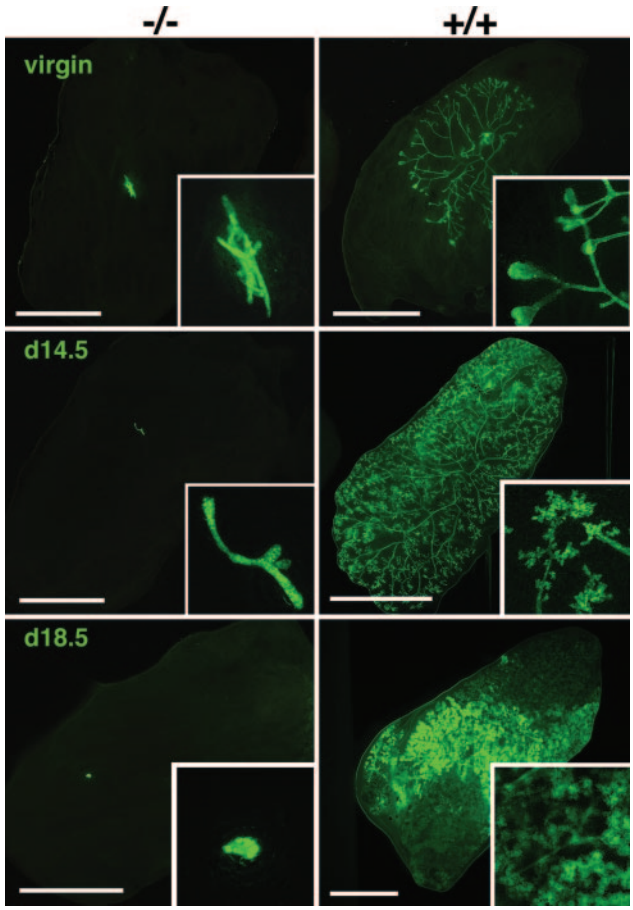

Fig. 2. The role of ERα in the mammary epithelium as shown by transplantation of epithelium. Fluorescent images of mammary glands from recipients at different developmental stages are shown. Preparations were derived from virgin (*Top*), day 14.5 (*Middle*), or day 18.5 (*Bottom*) pregnant recipients engrafted with ER$\alpha^{-/-}$ (*Left*) or WT (*Right*) epithelium. (Scale bars: 5 mm.) (*Insets*) Higher (×5) magnifications are shown.

Table 1. Requirement ERα in the mammary epithelium

Mice	No. with no detectable epithelium	No. with epithelial graft detected but no outgrowth	Total no. of mice
Virgin	7	13	20
Pregnant	2	6	8
Postpartum	2	6	8

This table summarizes the results of mammary gland reconstitution experiments with complete ERα$^{-/-}$ mammary epithelium. Contralateral control grafts showed normal development. At all developmental stages, no outgrowth was observed.

donors that expressed the GFP transgene ubiquitously (31) and visualized the epithelium under the fluorescence stereoscope. Fluorescent stereomicroscopy of grafted glands 5 weeks after surgery shows that WT epithelium grows out with large TEBs, whereas the ERα$^{-/-}$ epithelium completely fails to do so (Fig. 2 *Top*). Because of infertility, mammary gland development during pregnancy could not be assessed in the ERα$^{-/-}$ animals. We analyzed grafts in pregnant and postpartum recipient females. Even in the presence of the intense hormonal stimulation of pregnancy, ERα$^{-/-}$ epithelium remained rudimentary (Fig. 2). Overall, 36 successfully engrafted mice were analyzed (Table 1).

These findings were in contrast to previous transplantation studies with tissue from the incomplete ERα$^{-/-}$ mice, which suggested that hormonal stimulation elicited ductal elongation, side branching, and alveologenesis of ERα$^{-/-}$ epithelium (9). The apparent discrepancy may be attributable to the remaining ERα protein or stem from differences in experimental settings. Thus, in the previous experiments, both donor and recipient mice were treated with hormones. Furthermore, differences in chow and housing conditions could influence the experimental outcome. To eliminate the confounding factors, we transplanted mammary epithelium from the incomplete ERα$^{-/-}$ strain (25), adhering to the same experimental protocol as for the complete ERα$^{-/-}$ mammary epithelium. After engraftment, mice were left to recover and analyzed either as virgins or at different stages of pregnancy. In virgin mice, none of 12 ERα mutant grafts showed ductal outgrowth; pregnant recipients showed some development, increasingly so with more advanced pregnancy (Fig. 3 and Table 2). Thus, the remaining ERα protein suffices to mediate estrogenic activity in pregnant mice.

The Role of ERα in the Mammary Stroma. The observation that the epithelial ERα is essential for ductal outgrowth and subsequent morphogenetic steps is in contrast to previous work indicating that the stromal ERα has an important role in mammary gland development (9, 24). To assess the importance of stromal ERα, we grafted WT epithelium derived from ROSA26 mice (15) into ERα$^{-/-}$ mammary fat pads that we subsequently transplanted onto the abdominal muscle wall of WT recipients. ROSA26 transgenic mice express the β-galactosidase gene in virtually all their tissues (32). When the reconstituted fat pads were subjected to an X-Gal staining procedure, the implanted ROSA26-derived epithelium turned blue and could thus be unequivocally distinguished from any endogenous epithelium, which was visualized by the red color of the carmine/alum counterstain.

X-Gal staining and subsequent whole-mount microscopy revealed that, in virgin recipients, the ERα WT epithelium (Fig. 4, blue) was able to proliferate as indicated by the presence of prominent TEBs and ductal elongation (Fig. 4 *A* and *B*). In some of the samples, the endogenous ERα$^{-/-}$ epithelium (Fig. 4, red, arrow) could be observed. Consistent with the previous observations, the ductal tree failed to develop TEBs and remained rudimentary (Fig. 4 *A* and *B*, arrow). Analysis of mammary glands from recipients that were impregnated 8 weeks after the grafting showed that subsequent development in response to pregnancy hormones was not affected by the absence of the stromal ERα (Fig. 4 *C* and *D*).

We conclude that the mammary epithelium is the prime compartment of ERα signaling both before and during pregnancy and that a direct response of the mammary stroma to estrogens does not play an essential role.

The Role of ERα in Differentiation of the Mammary Epithelium. The results above establish a preeminent role for ERα in the mammary epithelium with regards to mammary proliferation and morphogenesis. To address whether ERα is also required for the differentiation program of mammary epithelial cells, we prepared histological sections of ERα$^{-/-}$ and WT epithelial grafts from hosts that gave birth. WT glands showed morphological hallmarks of secretory activity. The lumina of alveoli were filled with secretions, and extra- and intracellular fat droplets were observed, whereas, in ERα$^{-/-}$ epithelia, the ductal lumen was not distended, and the cells displayed no obvious morphological signs associated with secretory activity (Fig. 5*A*).

To address whether the ERα$^{-/-}$ epithelium is responsive to pregnancy-associated hormone stimulation, we determined mRNA expression of the genes encoding the milk proteins β-casein and α-lactalbumin. ERα$^{-/-}$ and WT epithelium were grafted to contralateral cleared fat pads of 3-week-old WT mice. Five weeks after surgery, the recipients were mated. At different days of late pregnancy, the part of the mammary gland containing the ERα$^{-/-}$ epithelium was dissected under UV illumination together with a

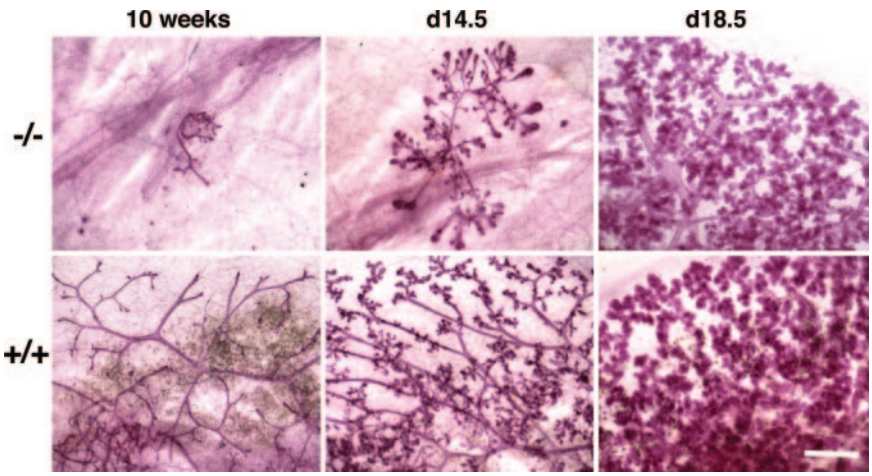

Fig. 3. Development of mammary epithelium expressing a truncated ERα as shown by transplantation of mammary epithelium from incomplete ERα$^{-/-}$ mice. Whole-mount preparations of mammary glands from WT recipients are shown. Preparations were derived from virgin (*Left*), day 14.5 (*Center*), or day 18.5 (*Right*) pregnant recipients engrafted with incomplete ERα$^{-/-}$ (*Upper*) or WT (*Lower*) epithelium. (Scale bar: 0.5 mm.)

Table 2. Development of incomplete ERα$^{-/-}$ mammary epithelium

Mice	No. with no outgrowth	No. with <10% outgrowth	No. with 10–95% outgrowth	No. with minor impairment	Total no. of mice
Virgin	12	0	0	0	12
Pregnant	10	4	11	1	26
Postpartum	5	1	9	6	21

This table summarizes the results of mammary gland reconstitution experiments with incomplete ERα$^{-/-}$ mammary epithelium. Contralateral control grafts showed normal development. Depending on the developmental stage of the host, the extent of outgrowth varied.

piece of mammary gland of comparable size dissected from the contralateral gland that had been engrafted with WT epithelium. Both pieces were processed for RNA extraction and subsequently analyzed by RT-PCR for expression of the epithelial marker keratin 18 for normalization and the genes encoding the two milk proteins β-casein and α-lactalbumin. mRNA expression of milk protein genes is induced in the ERα$^{-/-}$ epithelium (Fig. 5*B*). This finding indicates that the epithelial ERα is not absolutely required for the transcriptional response to pregnancy hormones.

ERα Acts in a Paracrine Fashion. Throughout development ERα is expressed only in a subset of MECs (33), indicating that not all of the epithelial cells are able to respond directly to estrogen. In the adult mammary gland, a dissociation of ERα-positive cells and BrdUrd-incorporating cells has been observed in different species (34–36). In the puberal mouse mammary gland, 66% of the proliferating cells show no expression of ERα (37). These findings are compatible with a scenario in which estrogen, like progesterone (7), acts by a paracrine mechanism to induce proliferation. Alternatively, cells may down-modulate expression of ERα when they proliferate. Consistent with the latter scenario is the observation that stimulation with estradiol leads to a decrease in ERα expression within 4 h (38).

To distinguish between these two models, we used a mixture of ERα$^{-/-}$ ROSA26 and ERα WT cells to reconstitute cleared fat pads. Tissue structures composed of ERα$^{-/-}$ cells would therefore turn blue upon X-Gal staining when analyzed by whole-mount microscopy. Structures composed of ERα WT cells would show the red of the counterstain.

Mixtures of ERα$^{-/-}$ and ERα WT MECs in a 1:10 ratio were injected into the cleared mammary fat pads of WT females. These mixtures were obtained by combining single-cell suspensions derived from ERα$^{-/-}$ ROSA26 and ERα WT females. The recipients were analyzed 5–10 weeks later. In 30 of 56 chimerical glands, we detected ERα$^{-/-}$ ROSA26 MECs (Fig. 6*A*). The mutant cells were found in all parts of the mammary glands, i.e., major ducts, TEBs, and side branches, indicating that estrogens act by a paracrine mechanism to induce proliferation.

The mammary epithelium consists of two major cell types: the luminal cells, which constitute the inner layer, and myoepithelial cells, which form the outer layer. To determine whether ERα$^{-/-}$ ROSA26 MECs contribute to both compartments, we assessed chimerical glands by histological sections. As shown in Fig. 6*B*, blue

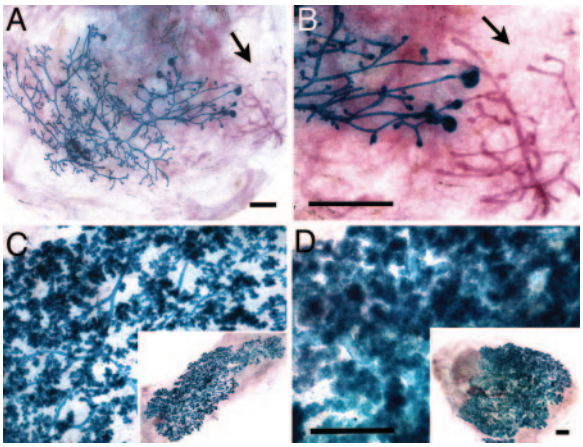

Fig. 4. The role of ERα in the mammary stroma. Whole-mount preparations of ERα$^{-/-}$ mammary glands engrafted with WT epithelium are shown in blue. Endogenous epithelium appears red (arrow). Reconstituted mammary glands were removed from the recipients stained with X-Gal before whole mounting. (*A* and *B*) Low magnification (*A*) and a more detailed view (*B*) of a gland derived from a virgin recipient. (*C* and *D*) Days 14.5 (*C*) and 18.5 (*D*) of pregnancy. (Scale bars: 1 mm.)

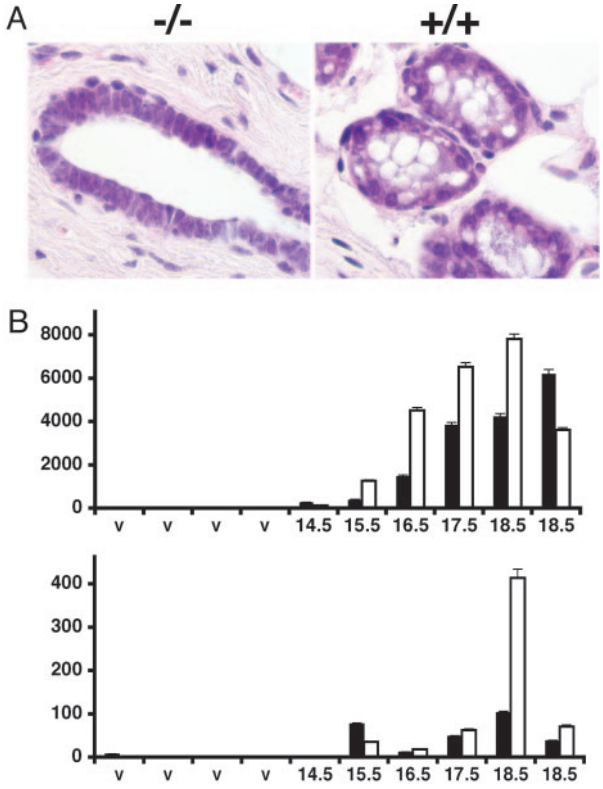

Fig. 5. The role of ERα in differentiation of the mammary epithelium. (*A*) Hematoxylin- and eosin-stained sections of mammary glands from a recipient engrafted with ERα$^{-/-}$ (*Left*) or WT (*Right*) epithelium postpartum. (*B*) Contralateral mammary glands from virgin (V) mice or mice at different days of pregnancy (days 14.5–18.5) engrafted with ERα$^{-/-}$ GFP^{+} and ERα$^{+/+}$ GFP^{+} epithelia were dissected under UV illumination. RNA was extracted, and cDNA was amplified with primers specific for *keratin18*, *β-casein* (*Upper*), and *α-lactalbumin* (*Lower*), and the products were quantified by real-time PCR in triplicate. The relative *β-casein* and *α-lactalbumin* expression after normalization for *keratin18* is shown. Solid bars indicate the glands engrafted with ERα$^{-/-}$ GFP^{+} epithelium. Open bars indicate the contralateral glands engrafted with ERα$^{+/+}$ GFP^{+} epithelium.

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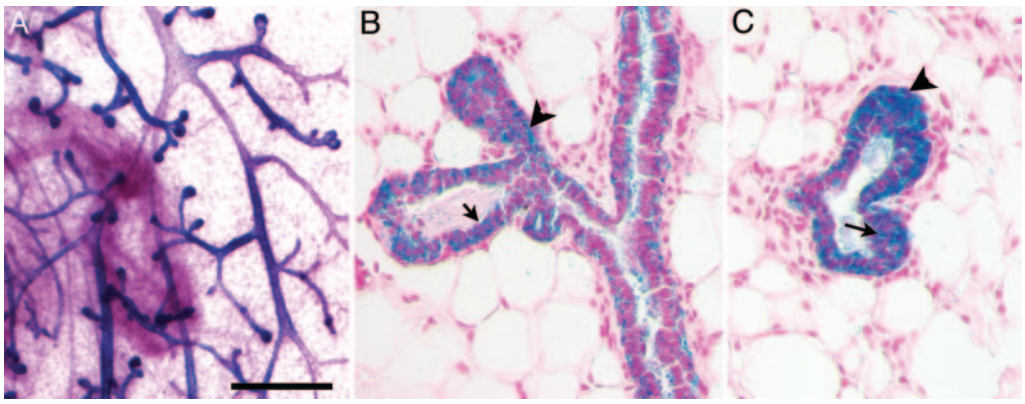

Fig. 6. Rescue of the ER$\alpha^{-/-}$ phenotype in ER$\alpha^{-/-}$ and ER$\alpha^{+/+}$ chimeric epithelia. (*A*) Whole-mount preparation of mammary fat pad injected with a mixture of ER$\alpha^{-/-}$ ROSA26 (blue) and ER$\alpha^{+/+}$ (red) epithelial cells in a 1:10 ratio after X-Gal staining is shown. (Scale bar: 2 mm.) (*B* and *C*) Histological section of the same gland counterstained with nuclear fast red. Note that ER$\alpha^{-/-}$ ROSA26 (blue) cells are found in both myoepithelial (arrowhead) and luminal (arrow) cell compartments (*B*) and are found in TEBs both in the cap cell layer (arrowhead) and among the body cells (arrow) (*C*).

cells are found both in the luminal and in the myoepithelial cell compartments. Moreover, ER$\alpha^{-/-}$ MECs can contribute to the outermost cell layer in the TEBs, consisting of the cap cells that are thought to give rise to both luminal and myoepithelial cells of the subtending duct (Fig. 6*C*). Thus, the presence of the ERα is required in only a portion of the MECs for ductal development to occur. Moreover, these findings suggest that estrogens activate a paracrine signaling route that operates between distinct subtypes of MECs, permitting ER$\alpha^{-/-}$ MECs to participate directly in ductal proliferation.

Discussion

Hormonal ablation/reconstitution experiments (3) have shown that estrogens are important in ductal outgrowth during puberty. It is thought that ERα signaling is important in this process; indeed, ablation of ERβ does not affect ductal growth (20). To determine the extent to which ERα signaling is limiting in development, we generated mice lacking the ERα gene (28). However, because the ER$\alpha^{-/-}$ females have multiple impairments in their reproductive functions, the specific consequences of ERα inactivation on mammary gland development could not be assessed in these mice. To circumvent this difficulty, we used various transplantation techniques to elucidate the role of estrogens in the development of the mammary gland and made use of cells derived from mice carrying a β-galactosidase or a GFP transgene to distinguish MECs of different genotypes.

Our experiments involving the transplantation of ER$\alpha^{-/-}$ MECs into cleared WT fat pads and WT cells into ER$\alpha^{-/-}$ fat pads were motivated by the need to assess the role of the ERα in both epithelial and stromal compartment *in vivo* under physiologic conditions. WT epithelium grafted into ER$\alpha^{-/-}$ fat pads developed normally, whereas ER$\alpha^{-/-}$ MECs did not grow at all, even when the host went through a series of estrous cycles and a normal pregnancy. This finding indicated that ductal proliferation and subsequent morphogenetic steps, i.e., side branching and alveologenesis, rely on the presence of ERα in the mammary epithelium and that other signaling mechanisms operating in the breast tissue cannot compensate for the absence of ERα. Our observation that epithelium from ERα mutant mice that express a protein lacking the transactivation function-1-containing N-terminal A/B region develops substantially during pregnancy suggests that the ligand-independent transactivation function may not mediate estrogenic activity during pregnancy.

We next addressed the issue of whether estradiol is required for differentiation of the mammary epithelium. To this aim we examined ER$\alpha^{-/-}$ MECs before and during pregnancy. We observed an induction of milk protein gene expression, indicating that although the ER$\alpha^{-/-}$ MECs completely fail to proliferate even during pregnancy, ERα signaling in the mammary epithelial cells is not absolutely required for transcriptional response to pregnancy hormones. Clearly, the amount of milk protein expression is lower than in the WT epithelium, and histological hallmarks of secretory activity are not discernable in the mutant epithelium. It is possible that ERα signaling may have a direct role in cytodifferentiation. Alternatively, the morphogenetic/structural changes may be tied to cytodifferentiation; hence, in the absence of ductal and alveolar morphogenesis, an inability to differentiate ensues. Alternatively, ERβ signaling may be important in mediating differentiation function. Transplantation experiments with double mutant epithelia should help to resolve this issue.

The precise mechanisms by which estrogens enable ductal MECs to proliferate leading to ductal outgrowth is unclear. The pattern of ERα expression in the mammary epithelium is heterogeneous (34, 37), suggesting the involvement of only a subset of ductal cells in estradiol-triggered processes. Our observation that ER$\alpha^{-/-}$ cells can proliferate indicates that estradiol does not need to act directly on MECs for them to participate in proliferation and morphogenesis. Thus, it appears that estradiol, like progesterone (7), acts on a subtype of ductal cells, causing them to release paracrine signals that permit other nearby epithelial cells, both luminal and myoepithelial, to participate directly in ductal outgrowth. Wnt-4 was identified as a paracrine mediator downstream of progesterone (39), and receptor activator of NF-κB-ligand (RANKL) has also been identified as a progesterone target and implicated in mediating progesterone-induced proliferation (40, 41). Prolactin relies on insulin-like growth factor (IGF) 2 as a mediator of its morphogenic effects (40). With regard to estradiol, amphiregulin is an attractive candidate for conveying the paracrine signal. Ablation of this growth factor in the mammary epithelium causes a phenotype similar to that of ERα mutant epithelium, and its transcription is strongly induced by estrogen (L. Ciarloni and C.B., unpublished observations). Interestingly, the receptor for amphiregulin, the epidermal growth factor receptor (EGFR), is required in the fat pad (42), suggesting that the estrogen-induced paracrine-signaling loop involves the stroma. In response to amphiregulin, stromal cells in turn may release fibroblast growth factors (FGFs) that act on the epithelial cells (43).

The observation that proliferating cells in the adult mammary epithelium rarely express steroid receptors was made in mice, rats, and humans (34–36), suggesting that estrogens and progesterone generally operate by paracrine mechanism in the breast. Intriguingly, in ERα-positive human breast carcinomas, which represent 2/3 of all breast cancers, ERα-positive cells proliferate (34). This proliferation may be a reflection of increased paracrine growth stimulation. Alternatively, the rare population of ERα positive proliferating cells (37) expands as an ERα-positive tumor develops or ERα-positive cells acquire the ability to use estrogen as a direct mitogen. ERα-negative tumors, however, may escape the requirement for paracrine growth stimulation by steroid hormones by constitutively activating growth factor signaling pathways. Consis-

tent with this model, epidermal growth factor receptor overexpression is frequently associated with ERα-negative tumors (44). The use of tissue reconstitution techniques and genetically altered cells should help to further dissect the intercellular communication involved in mammary morphogenesis and carcinogenesis.

Methods

Mice. ROSA26 mice were purchased from The Jackson Laboratory. The ERα mutant mice were described elsewhere (25, 28). All mice were bred in C57BL6 or 129SV/C57BL6 genetic background. Presence of the β-galactosidase transgene was tested for by subjecting a piece of tail to the X-Gal staining procedure described below.

Whole-Breast Transplant. Four- to 6-week-old $ER\alpha^{-/-}$ female mice were killed, inguinal and thoracic mammary glands were dissected, and each gland was engrafted with a piece of mammary tissue of 1-mm diameter from ROSA26 females. Two-month-old $(129SV/C57BL6)F_1$ females were anesthetized with isoflurane and burprenorphine s.c. The ventral skin was incised, and the abdominal muscle wall was exposed. A recombined gland was placed onto the abdominal wall, and the incision was closed with a surgical staple. Some of the recipients were killed 4 weeks after surgery, others were killed during pregnancy. The transplanted gland and an endogenous mammary gland were analyzed by whole-mount microscopy.

Transplantation of Mammary Epithelium. The fat pads of 3-week-old C57BL6 or 129SV/C57BL6 females were cleared. Pieces of mammary tissue of 1-mm diameter were removed from the nipple region of $ER\alpha^{-/-}$ GFP^+ and $ER\alpha^{+/+}$ GFP^+ females under the UV lamp to ensure the presence of epithelium and implanted as described in ref. 7. Alternatively, the cleared fat pads were injected with $ER\alpha^{-/-}$ GFP^+ and $ER\alpha^{+/+}$ GFP^+ primary MECs, which were cultured as described in ref. 40.

Mammary Gland Whole Mounts. Mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in 1:2 benzyl alcohol/benzyl benzoate (Sigma) as described in ref. 9. Digital pictures were taken on a Leica (Heerbrugg, Switzerland) MZFLIII stereoscope or Leica DM2000 microscope with Leica DC300F and PixeLINK PL-A622C, respectively.

X-Gal Staining. The transplanted mammary glands were dissected, fixed for 1 h in 1.5% formaldehyde in PBS, washed three times over 3 h with rinse buffer (2 mM $MgCl_2$/0.1% sodium deoxycholate/0.2% Nonidet P-40 in PBS, pH 7.4) and rotated in X-Gal staining solution (1 mg/ml X-Gal/5 mM potassium ferricyanide/5 mM potassium ferrocyanide in rinse buffer) at 37°C for 18 h, washed in PBS, and processed for whole mounting as described above.

Quantitative Real-Time PCR (QRT-PCR). Total RNA was reverse transcribed by using reverse transcriptase (GIBCO/BRL) and random hexamers (Roche, Rotkreuz, Switzerland). The resulting cDNAs were used for quantitative PCR analysis using the iCycler apparatus (Bio-Rad) and SYBR Green PCR Core Reagents system (Qiagen, Hombrechtikon, Switzerland). Results were evaluated with ICYCLER IQ REAL TIME DETECTION SYSTEM software (Bio-Rad). The following primers were used: *β-casein* forward, CTTCAGAAGGTGAATCTCATGGG; *β-casein* reverse, CAGATTAGCAAGACTGGCAAGG; *α-lactalbumin* forward, ACCAGTGGCTACGACACAC; *α-lactalbumin* reverse, CGGGGAACTCACTACTTTTACAC, and *keratin18* (45).

Histological Examination and BrdUrd Immunohistochemistry. For histological examination, whole-mounted mammary glands were washed in 100% ethanol before paraffin embedment. Sections were cut at 4 μm. Mice were injected with BrdUrd 2 h before they were killed. Anti-BrdUrd (OBT0030, Oxford Biotechnology, Oxfordshire, U.K.) was diluted 1:300 and applied overnight at 4°C after antigen retrieval in citrate buffer. Biotinylated secondary antibodies were detected with a VECTASTAIN Elite kit (Vector Laboratories).

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